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Binding affinity of *Plasmodium falciparum*-infected erythrocytes from infected placentas and laboratory selected strains to chondroitin 4-sulfate

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Abstract

The adherence of *Plasmodium falciparum*-infected red blood cells (IRBCs) in human placenta is mediated by chondroitin 4-sulfate (C4S). The C4S-adherent parasites selected from laboratory strains have been widely used for determining the C4S structural elements involved in IRBC binding and for the identification of parasite adhesive protein(s). However, the relative binding strength of the placental versus laboratory-selected parasites has not been reported. In this study, we show that IRBCs from the infected placentas bind to C4S about three-fold higher than those selected for C4S adherence from laboratory strains. Although adherent parasites selected from several laboratory strains have comparable binding strengths, the one obtained from a 3D7 parasite clone termed, 3D7N61 used for malaria genome sequencing, exhibits markedly lower binding strength. Furthermore, 3D7N61-CSA parasites lose most of the binding capacity by tenth generation in continuous culture.

Keywords

Plasmodium falciparum; infected erythrocytes; adherence; chondroitin 4-sulfate; laboratory parasite strains; placental parasite isolates; binding strength

A unique feature of *Plasmodium falciparum* infection in pregnant women is that parasite-infected red blood cells (IRBCs) sequester extensively in the placenta, causing placental malaria that is associated with poor pregnancy outcomes and severe maternal anemia and death [1–3]. While CD36 on the endothelial cell surface is the major receptor for IRBC sequestration in the microvascular capillaries [2,4], chondroitin 4-sulfate (C4S) chains of uniquely low sulfated chondroitin sulfate proteoglycans (CSPGs) mediate placental IRBC accumulation [3,5–7]. Developing a vaccine for placental malaria based on disrupting C4S-IRBC interaction, thereby preventing placental IRBC accumulation using the parasite adhesive protein as a candidate has been extensively pursued [1,8]. However, a critical requirement for such efforts is the definitive identification of parasite adhesive protein(s) expressed on the IRBC surface. Although a number of studies have reported DBL- γ domain of *var1* subfamily *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) as the ligand for IRBC binding to C4S [2,9,10], recent studies suggest that DBL-like domains of *var2* subfamily PfEMP1 mediates the cytoadherence [8,11,12]. However, in both cases, involvement of PfEMP1 in IRBC adhesion has not been conclusively established. A scenario wherein novel proteins unrelated to PfEMP1

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playing roles in the adhesive process cannot be ruled out. Consistent with these predictions, recent studies implicate novel proteins in IRBC adherence [13–16]. Thus, the development of a vaccine for placental malaria remains challenging.

A promising alternative approach for the treatment of placental malaria would be to develop inhibitors that efficiently disrupt C4S-IRBC adherence. In this regard, either structurally defined C4S oligosaccharides or rationally designed peptide mimetics might prove to be effective therapeutic agents. Such efforts require a comprehensive understanding of the structural interactions involved in C4S-IRBC binding. Although recent studies have provided a considerable amount of information on the critical structural elements involved in C4S-IRBC adhesion [7,17–19], further understanding of the precise sulfate group distribution required for maximal IRBC binding and determination of binding strength are also critically needed. Studies aimed at delineating the structural elements involved in C4S-IRBC binding have used C4S-adherent parasites selected from different strains of laboratory-cultured parasites. The adherent parasites were obtained by panning laboratory strains either on immobilized bovine tracheal C4S or CSPG purified from human placenta. Recently, a human choriocarcinoma trophoblast cell line, BeWo, has been used for selecting C4S-adherent parasites [20–22] and found that the selected parasites bind predominantly to CSA, although minor populations exhibited binding specificity to other ligands. However, it should be noted that CSPG synthesized by cultured BeWo cells have not been characterized and it is possible that CSPGs synthesized by these cells are markedly different from those present in human placenta. Some studies have noted that the adherent parasites selected from various strains exhibit different binding capacity (6,23). However, the reasons for the observed differences in binding ability of selected parasites from various laboratory strains remain unclear. Possible reasons are: (i) the selected parasites might be phenotypically different, expressing polymorphic adhesive protein(s) or they could express different levels of adherent protein(s) on the IRBC surface. (ii) The differences could be simply a reflection of variations in the binding strengths with which the parasites were selected on structurally distinct receptors used for panning; for example, the bovine tracheal CSA, an unnatural receptor, is structurally different from C4S chains of the placental intervillous CSPG, a natural receptor [24]. (iii) The binding studies might have been performed with parasites cultured for different duration after panning; it is important to assess the C4S-IRBC interactions soon after panning because their binding strengths drastically decreases after prolonged culturing [25]. To rationalize the results obtained using C4S-adherent parasites from various laboratory strains for therapeutic applications, it is essential to understand the differences and similarities in binding strength of these parasites as compared to those of placental isolates. Therefore, relative evaluation of variously selected parasites and those isolated from infected placenta is required. In this study, we focused to comparatively assess the binding strengths of a panel of C4S-adherent laboratory strains by panning on CSPG purified from human placenta and two parasite isolates obtained from infected placentas. All IRBC binding and inhibition studies were performed using the placental CSPG, the receptor that mediates *in vivo* IRBC adherence.

The relative binding strength of laboratory C4S-selected parasites versus placental isolates was assessed by measuring the amount of a partially sulfated C4S polysaccharide required for inhibition of IRBC binding to human placental CSPG. The proteoglycan was isolated from the isotonic buffer extracts of freshly delivered placentas, collected from the Medical Center Maternity Ward, by DEAE-Sephacel column chromatography using NaCl gradient elution as described previously [5]. The CSPG was purified by CsBr density gradient centrifugation followed by gel filtration on Sepharose CL-6B [5]. The CS chains of the purified CSPG consists of ~8% 4-sulfated and ~92% non-sulfated disaccharides. This CSPG is comparable to the previously reported low sulfated CSPG present in the intervillous space and could specifically and efficiently bind IRBCs from both placental isolates and C4S-selected laboratory parasites [5]. Laboratory parasite strains were cultured using O-positive human erythrocytes and O-

positive human plasma and the C4S-adherent IRBCs were selected by panning on plastic plates coated with 250 ng/ml CSPG in PBS [17]. The IRBC binding assays were performed by allowing IRBCs to bind to the purified human placental CSPG coated on plastic plates at different concentrations and counting the IRBCs bound to unit area of the plates under microscopy. Inhibition assays were performed by allowing IRBCs pretreated with different concentrations of C4S to bind to plastic plates coated with 250 ng/ml of the purified placental CSPG [17]. The extent of inhibition was assessed, using light microscopy, by measuring the IRBCs bound to CSPG at each C4S inhibitory concentration. The partially sulfated C4S polysaccharide used for inhibition assays consisted of 36% 4-sulfated and 64% nonsulfated disaccharides and was prepared by regioselective 6-*O*-desulfation of bovine tracheal CSA (Sigma) followed by fractionation on DEAE-Sephacel column as reported previously [17, 24]. We have previously shown that this partially sulfated C4S fraction effectively inhibits IRBC binding to the human placental CSPG [17,24]. All inhibition assays were performed three times each in duplicates. Statistical analysis of data was performed using GraphPad Prism 4.0. Variations between two groups were determined by Student 't' test. Two-way ANOVA was used to analyze differences among group means. P values of ≤ 0.05 was considered significant.

The laboratory *P. falciparum* strains assessed in this study were those previously used by various investigators for studying C4S-IRBC interactions. These include, C4S-selected parasites from a 3D7 clone derived from NF54, FCR3, CS2 parasite strains [10,17], and the 3D7N61 clone that was used for the parasite genome sequencing. The C4S-adherent parasites were selected by 5–6 rounds of panning, each time after culturing for 4–5 generations, on plastic Petri dishes coated with the placental CSPG; further rounds of panning did not enhance the binding capacity (not shown). The C4S-selected laboratory parasites were designated as 3D7-CSA, FCR3-CSA, CS2-CSA, and 3D7N61-CSA. To determine the IRBC binding ability and binding strengths by adherence and adherence inhibition assays, the selected laboratory parasites were analyzed at the early trophozoite stage because parasites at this stage exhibit maximum adherence capacity [25]. Since we have previously shown that the binding capacity of 3D7-CSA and FCR3-CSA decreases gradually over successive generations after selection [25], the second-generation parasites after selection were used in this study. To determine the binding characteristics of 3D7N61-CSA parasites at various generations after C4S-selection, IRBCs from continuous culture were used. The placental parasite isolates were obtained from two *P. falciparum*-infected Cameroonian women placentas. The parasites from infected placentas were cultured for one generation (~10% parasitemia), harvested at the early trophozoite stage as described previously [26], and stored frozen in glycerolyte solution at -80°C until used. The two placental isolates were not genotyped and therefore, it is not known whether these isolates are genetically identical or diverse.

The C4S-adherent laboratory parasites and placental isolates, adjusted to ~10% parasitemia, were assessed for their binding capacity. In binding assays, both laboratory selected C4S-adherent and placental parasites bound to the CSPG in a coating-concentration dependent manner (Fig. 1A). In the case of laboratory parasites, 3D7-CSA and FCR3-CSA, the binding was saturated at 125 ng/ml of CSPG coating concentration with ~2400–2600 IRBCs binding per mm^2 . The level of IRBC binding by 3D7N61-CSA parasites was substantially lower than the other laboratory parasites tested and the binding was saturated at a coating concentration of 250 ng/ml CSPG with ~1500 IRBCs binding per mm^2 (Fig. 1A). On the other hand, the placental parasites showed saturated level of binding at 62.5 ng/ml of CSPG coating concentration with ~2700–2800 IRBCs binding per mm^2 . Thus, placental isolates could bind more efficiently to placental CSPG than the laboratory strains. No significant difference was found between the two placental isolates ($p > 0.05$). However, significant differences were observed in the binding capacity between each placental isolate and three laboratory strains, FCR3-CSA, 3D7-CSA and 3D7N61-CSA; p values were, respectively, < 0.05 , < 0.01 and

<0.001 (Fig. 1A inset and data not shown). The laboratory parasites, FCR3-CSA, 3D7-CSA, and 3D7N61-CSA, also differed significantly from each other in their binding capacity ($p < 0.05$ or < 0.01).

Since the IRBC binding assay does not measure the avidity of IRBC binding to the CSPG, the relative binding strengths of the laboratory selected and placental parasites were determined by measuring the inhibition of IRBC binding to placental CSPG using the partially sulfated C4S polysaccharide as reported previously [17,25]. The soluble C4S inhibited IRBC binding of the C4S-selected laboratory strains and placental isolates in a dose-dependent manner (Fig. 1B). The inhibitory capacity of the C4S, which reflects the binding strength of IRBC adhering to the placental CSPG, was substantially different for placental and laboratory parasites. However, the binding strengths of the two placental isolates were not significantly different from one another, $p > 0.05$ (Fig. 1B inset). The concentration of C4S required for inhibition of placental IRBC binding to CSPG by 50% (IC_{50}) was comparable for both isolates and it was about $\sim 35 \mu\text{g/ml}$ (Fig. 1B). In contrast to placental parasites, IC_{50} for the inhibition of FCR3-CSA, 3D7-CSA or CS2-CSA IRBCs binding to placental CSPG was $9\text{--}11 \mu\text{g/ml}$. Thus, the strength of placental parasites binding to the CSPG is ~ 3.5 -fold higher than that of IRBCs from commonly used laboratory C4S-selected strains. In the case of 3D7N61-CSA parasites, the binding strength of IRBCs was >10 -fold lower than that of each placental isolate and 3- to 4-fold lower than those of other laboratory parasites tested (Fig. 1B).

The observed difference in the binding strengths of various parasite strains could be due to either genetic polymorphisms in parasite protein(s) that mediates IRBC adherence to placental CSPG or variation in the level of expression of highly conserved adhesive protein(s) on IRBC surface. Previously, IRBC adhesion-inhibition studies using plasma from pregnant women in different endemic areas of the world have predicted conserved adherent protein among parasites from geographically different endemic areas to mediate IRBC binding to C4S [27]. However, recent studies that implicate *var2* PfEMP1 as the C4S-adhesive protein seem to disagree with this notion based on the conclusion that *var2* PfEMP1 mediates C4S-IRBC binding and that genetically variant parasites express polymorphic *var2* PfEMP1 [28,29]. Further, it has been suggested that different parasite strains, expressing polymorphic *var2* PfEMP1, interact differentially with structurally distinct C4S motifs differing in chain length and sulfate contents [29]. However, alternatively, the differences in that study could be due to the very low binding strengths of IRBCs used ($\sim 0.5 \mu\text{g/ml}$ bovine tracheal CSA was sufficient for complete inhibition) and/or due to a variable duration of culturing continuously after selection of adherent parasites. Detailed biochemical studies and functional analyses using placental CSPG and IRBCs selected for high binding strengths are needed to determine whether the observed differences in binding strengths of C4S-selected parasites are due to *var2* PfEMP1 polymorphism or to variation in the expression level of adhesive protein(s) on the IRBC surface.

We have recently shown that the levels of FCR3-CSA and 3D7-CSA IRBC binding as well as their binding strengths gradually decrease over successive generations in continuous culture following selection for C4S-binding [25]. After 32 generations of continuous culture, without subsequent selection, the binding strength of C4S-selected IRBCs from 3D7-CSA and FCR3-CSA decreased by $\sim 75\%$. Based on these results, it was suggested that the expression of adhesive protein(s) on IRBCs decreases gradually over successive generations and, thus eventually, parasites might become essentially nonadherent [25]. Since 3D7N61-CSA parasites selected by several rounds of panning on placental CSPG exhibit 3–4 fold lower binding strength compared to similarly selected 3D7-CSA and FCR3-CSA, it would be insightful to determine whether 3D7N61-CSA parasites also lose their binding strengths gradually over successive generations during continuous culture. Therefore, we measured the binding characteristics of 3D7N61-CSA at different generations after selection for C4S

adhesion and compared with the data for FCR3-CSA and 3D7-CSA obtained in parallel. As in the case of 3D7-CSA and FCR3-CSA parasites [25], the binding strength of 3D7N61-CSA decreased over successive generations (Fig. 2A). However, the binding strength of 3D7N61-CSA declined much more rapidly over successive generations; by tenth generation, ~77% of its binding strength was lost (Fig. 2B and 2C). In contrast, by tenth generations only ~23% and ~30% of the initial binding capacity of FCR3-CSA and 3D7-CSA was lost (Fig. 2B and 2C). As suggested previously [25], this gradual loss of C4S-IRBC binding strengths during successive generations in continuous culture is likely due to the gradual decreased expression of adhesive protein(s) on the IRBC surface [25]. Alternatively, as reported recently by others [29], *var2* PfEMP1 polymorphism might contribute to variation in IRBC binding strengths of parasite strains. However, it should be noted that the level of *var2* PfEMP1 expression on the IRBC surface could vary in a strain dependent manner and that its expression could decrease over successive generations. Furthermore, although recent studies have raised monoclonal and polyclonal antibodies against *var2* PfEMP1 [30,31], so far the ability of antibody to block IRBC binding to C4S has not been demonstrated, suggesting that *var2* PfEMP1 might not be directly binding to C4S. Further studies are required to determine whether *var2* PfEMP1 mediates IRBC binding. If *var2* PfEMP1 alone is not found to be sufficient for C4S binding, then the identification and characterization of the parasite adhesive protein(s) remain challenging.

Our data show that placental parasites bind much more strongly to the placental CSPG receptor compared to the laboratory C4S-adherent parasites, despite the latter being selected for maximum binding by several rounds of panning using placental CSPG. This is likely due to the fact that, *in vivo*, parasites express adhesive proteins at higher levels on the IRBC surface as compared to cultured parasites. This conclusion is consistent with the known information that, *in vivo*, parasites express higher levels of knobs with which the adhesive proteins are associated [32]. Thus, we predict that the low binding strength of laboratory selected parasites, especially 3D7N61-CSA analyzed here as well as HB3-CSA and HCS3-CSA parasites previously studied by others [6,23], as compared to placental isolates is likely due to relatively low levels of knobs on the IRBC surface of these laboratory isolates. This suggestion is supported by our recent observation that the levels of expression of knob-associated protein genes, KAHRP, PfEMP3 and *var2*-CSA, measured by real time RT-PCR, were markedly lower in 3D7N61-CSA parasites as compared to those of 3D7-CSA or FCR3-CSA (our unpublished results). The level of KAHRP in 3D7N61-CSA, measured by indirect immunofluorescence using anti-KAHRP antibody, was also considerably lower as compared to 3D7-CSA or FCR3-CSA. Our observations point to an interesting scenario in *in vivo* situations. If in fact the binding strength of placental CSPG-selected parasites decreases gradually *in vivo*, after several generations, the daughter parasites gradually lose their affinity to bind to the placenta and eventually enter into the circulation. However, the high affinity binding parasites that robustly express adherent protein(s) on the IRBC surface are selected continuously by the CSPG in placenta and they efficiently colonize the placenta until the adhesion inhibitory antibodies are produced. Thus, IRBCs isolated from *P. falciparum*-infected placentas are likely to have relatively high C4S-binding strengths.

Taken together our data also point to the following: (i) Since C4S adherent parasites selected from various laboratory strains differ substantially from one another in their binding strengths, parasites must be pre-assessed for their binding strengths and those with highest binding strengths should be used. Considering that binding strength of the C4S adherent parasites decreases gradually over successive generations, parasites used for binding studies should be freshly selected for maximal binding capacity by several rounds of selection, preferably by panning on placental CSPG. Furthermore, since the early trophozoite stage parasites bind more strongly than late stage parasites [25], the former are preferable. (ii) Ideally, parasites obtained from infected placenta are preferable for studying C4S-IRBC interactions. Because of practical

difficulties in obtaining placental parasites, laboratory strains remain source of choice for studying C4S-IRBC interactions. However, caution must be exercised in choosing laboratory strains; as far as possible, parasites that exhibit high binding strengths should be preferred. This is not only important in studying the effect of the adhesive protein gene polymorphism and in determining fine structural elements in C4S-IRBC interactions, but also to assess the efficacy of inhibitors/therapeutics required for the efficient disruption of IRBC binding to human placenta. (iii) Because the placental parasites differ significantly in their binding strengths and perhaps also in other characteristics as compared to adherent parasites selected from laboratory strains, care must be taken in reaching conclusions, based on the results obtained using laboratory parasites, regarding the efficacy of inhibitors in disrupting the C4S-IRBC interactions.

In conclusion, the data presented here show that IRBCs from *P. falciparum*-infected placentas bind placental CSPGs at much higher avidity compared to laboratory isolates. Among C4S-binding parasites selected from different laboratory strains, the binding strengths vary considerably. Compared to FCR3-CSA and 3D7-CSA parasites, the binding capacity and binding strength of 3D7N61-CSA IRBCs decreases more rapidly during successive generations, and that most of their binding affinity to placental CSPG was lost by tenth generations. This is likely due to the gradual decreased expression of C4S-adhesive protein(s) on the IRBC surface. Therefore, care must be taken in choosing the laboratory parasite strains for studying C4S-IRBC interactions, and parasites should be panned several rounds on placental CSPG and should be washed thoroughly to remove the weakly adhered IRBCs. Furthermore, parasites must be reselected after several generations in continuous culture.

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Fig. 1.

The binding efficiency of placental and laboratory selected *P. falciparum* IRBCs to placental CSPG. Parasite culturing, IRBC selection using the placental CSPG, and binding and inhibition assays were performed as reported previously [17]. Placental isolates were obtained as outlined previously [26]. (A) The binding of *P. falciparum* IRBCs to the purified human placental CSPG. Two placental isolates and four laboratory strains, harvested at early trophozoite stage and all adjusted to ~10% parasitemia, were assessed for binding to plastic plates coated with the indicated coating concentrations of the purified human placental CSPG. The number of IRBCs bound per mm² of CSPG-coated spots was assessed under light microscopy. Values shown are the mean of three independent experiments performed in duplicates. The vertical

bars indicate standard errors of the mean. The binding capacity (number of IRBCs bound per mm² of CSPG-coated plates) of placental isolates is significantly higher than those of laboratory selected parasites. (B) The relative strengths of *P. falciparum* IRBCs binding to placental CSPG. The relative strength of IRBC binding to human placental CSPG was determined by measuring the dose-dependent inhibition of binding by C4S. The binding of IRBCs pretreated with C4S was performed as outlined in panel A. The binding strength of placental IRBCs, as measured by the concentrations of soluble C4S required for 50% inhibition of IRBC binding to CSPG, is considerably higher as compared to IRBCs selected from laboratory parasite strains. In both panels: ● and ○ represent IRBCs from infected placentas 1 and 2, respectively; ▲, △, and □ correspond to laboratory selected 3D7-CSA, FCR3-CSA, and 3D7N61-CSA parasites, respectively. CS2-CSA parasites were also tested and the values were similar to that of FCR3-CSA parasites (not shown). Inset in panel A shows the relative capacity of IRBC binding to placental CSPG coated to plastic plates using 32 ng/ml solution. Inset in panel B represents inhibition of the binding of placental and laboratory parasite IRBCs to placental CSPG by the soluble C4S. In both insets: hatched bars, placental isolate 1; black bar, placental isolate 2; grey bar, FCR3-CSA; bar with horizontal lines, 3D7-CSA; open bar, 3D7N61-CSA. Statistical significance between samples was evaluated by Student 't' test. NS, not significant, *, p <0.05; **, p <0.01; ***, p <0.001.

Fig. 2.

The CSPG-binding strengths of 3D7N61-CSA *P. falciparum* IRBCs from successive generations during continuous culturing. (A), The relative strength of IRBC binding to human placental CSPG was measured by determining the dose-dependent inhibition of binding by the partially sulfated C4S having 36% 4-sulfate. Inhibition assay using IRBCs from different parasite generations in continuous culture after selection for maximum IRBC binding capacity was performed as outlined in Fig. 1B. The number of IRBCs bound per mm^2 with control untreated samples was in the range of 1400–2800. The plotted values represent percent IRBCs bound per mm^2 compared to samples not treated with C4S and are mean of three independent experiments done in duplicate. Values are plotted as mean $\pm$ standard errors of the mean. The

graph shows the inhibition of IRBCs from second (●), fourth (○), seventh (▲) and tenth (△) generations binding to CSPG by soluble C4S. (B), The binding strength of 3D7N61-CSA IRBCs (IC_{50} , the concentration of C4S required for 50% IRBC binding to the placental CSPG) as compared to that of FCR3-CSA IRBCs. When both parasites measured at the second and tenth generations after selection, the binding strength of 3D7N61-CSA was 26% and 6% of those of FCR3-CSA. (C), The binding strength of 3D7N61-CSA IRBCs ($C4S\ IC_{50}$) as compared to 3D7-CSA IRBCs. At the second and tenth generations after selection, the strength of 3D7N61-CSA was 31% and 8% as compared to 3D7-CSA.